THE OXIDATIVE METABOLISM OF EGGS OF URECHIS CAUPO [1]

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The cytochrome system is of such widespread occurrence in cells of aerobic organisms that reports of its absence in any particular case are of special interest.

There have been several instances in which spectroscopic detection of absorption bands of the cytochromes was at first reported to be negative and later shown to be positive when improved methods were employed. Thus in eggs of sea urchins Brachet (1934), Lindahl (1936), Krahl, Keltch and Clowes (1939) and Ball and Meyerhof (1940) reported that the cytochrome bands did not show up spectroscopically, although early evidence of inhibition of O_2-uptake by cyanide and by carbon monoxide (Runnström, 1930) indicated the operation of the cytochrome system, at least in the fertilized eggs. Later, Rothschild (1949), Borei (1951) and Yčas (1954), using the method of Keilin and Hartree (1939, 1949) of intensifying cytochrome bands by cooling the material in liquid air, were able to demonstrate the bands of cytochromes *a* and *b*.

It was also thought, at one time, that the respiratory system differed qualitatively in unfertilized and fertilized sea urchin eggs, the cytochrome system being inoperative in the former and brought into play upon fertilization (Korr, 1939). This was based on evidence of insensitivity of the respiration of unfertilized eggs to inhibition by cyanide and carbon monoxide (Runnström, 1930; Lindahl, 1939; Korr, 1937), and reported differences in the effect of temperature on the respiration of unfertilized and fertilized eggs (Rubenstein and Gerard, 1934; Korr, 1937). However, this evidence has now been largely contradicted. Thus, Robbie (1946b) showed that the O_2-uptake of unfertilized sea urchin eggs could be almost completely inhibited by low concentrations of cyanide when the precaution is taken of preventing the distillation of cyanide from the egg suspension to the center-well of the manometer vessel, by the use of appropriate $Ca(CN)_2$–$Ca(OH)_2$ mixtures in the center well. In regard to the effect of carbon monoxide on unfertilized sea urchin eggs, Rothschild (1949) was able to demonstrate a photo-reversible inhibition of O_2-uptake when account was taken of a CO-induced stimulation and a light-induced inhibition of respiration. Concerning the effect of temperature on respiratory rates, further measurements (Tyler and Humason, 1937; Borei and Lybing, 1949) have shown no significant differences between unfertilized and fertilized sea urchin eggs.

In eggs of the echiuroid worm *Urechis caupo*, a failure to detect the absorption bands of cytochrome was reported by Horowitz and Baumberger (1941). In these eggs there is a reversibly autoxidizable pigment which Horowitz (1940a)

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called urechrome. From the facts that both the oxidized and reduced states are observed naturally in the eggs, depending upon the presence or absence of oxygen, that the pigment is reducible by the cells, and that it autoxidizes in the physiological range of pH, he concluded that it was probably involved in the cellular respiration. Upon further characterization Horowitz and Baumberger (1941) suggested that the pigment was related to the hemins. Its chemical constitution has not as yet been determined.

For these and other reasons we decided to examine the O_2-uptake of fertilized eggs of *U. caupo* in the presence of cyanide and of carbon monoxide, and to examine the eggs spectroscopically using the low temperature method of Keilin and Hartree (1939). Previous measurements of the respiration of this material were made for other purposes (Tyler, 1936; Tyler and Humason, 1937; Tyler and Horowitz, 1938; Horowitz, 1940b) and it was noted that the fertilized eggs have rather consistent values for their absolute rate of O_2-uptake, although that of the unfertilized eggs may vary greatly from one batch to another. Some preliminary experiments with cyanide and azide were mentioned in the report of a seminar talk (Tyler, 1937). These were for the purpose of investigating possible correlations between cleavage retardation and respiratory inhibition and were done before the introduction by Robbie (1946a) of the $Ca(CN)_2$–$Ca(OH)_2$ center-well mixtures for preventing loss of cyanide from cell-suspensions in the manometer vessels. The preliminary experiments indicated, however, that inhibition of respiration by cyanide was obtainable in these eggs.

Material and Methods

General manometric procedure. Eggs of *U. caupo* were inseminated and washed in sea water buffered at pH 8 with 0.01 *M* glycylglycine, which Tyler and Horowitz (1937) showed to be a suitable non-injurious agent to replace the bicarbonate system of ordinary sea water. The latter system does not provide satisfactory buffering because the absorption of CO_2 by the alkali in the manometer vessels occasions a rise in pH of the sea water which is only partially and variably compensated by the CO_2 production of the respiring cells.

After examination to check that fertilization had been successful, 3- or 4-ml. aliquots of egg-suspension were transferred to standard Warburg-Barcroft manometer flasks whose calibration volumes were around 20 ml. Readings were taken after a 30-minute equilibration period in the water-bath, the shaker speed being 95 c.p.m. at 4 cm. stroke. The temperature was 20° C.

Cyanide experiments. Robbie's (1946a) $Ca(CN)_2$–$Ca(OH)_2$ mixtures, in 0.6-ml. quantity, were used in the center-wells of the manometer vessels in order to establish and maintain known concentrations of cyanide in the egg suspensions, and provide sufficient alkali to absorb the respiratory CO_2. Fluted filter papers were used in the center-wells to increase the absorbing surface. A stock 1.32 *M* calcium cyanide solution was prepared according to Robbie and Leinfelder (1945) and this was diluted with 10% $Ca(OH)_2$ according to Robbie's (1946a) figures to provide center-well mixtures establishing the following concentrations of HCN in the experimental fluid at 20° C.

HCN molarity	10^{-3}	10^{-4}	5×10^{-5}	10^{-5}	5×10^{-6}
Molarity of $Ca(CN)_2$ in 10% $Ca(OH)_2$	0.38	0.046	0.023	0.0054	0.0028

In some experiments an appropriate quantity of NaCN was added to the egg-suspensions in the manometer flasks just before the beginning of the experiment. In the latter case the equilibration-time was reduced from thirty to fifteen minutes. Manometer flasks and other vessels containing cyanide solutions were kept stoppered at all times except when eggs were added and the flasks were put on the manometers.

CO experiments. The gas phase of the manometers was filled with 95% CO in O_2 (95% CO/O_2), after flushing out the air. Ninety-five per cent N_2/O_2 and air controls were run at the same time. The center wells contained 0.3 ml. N/1 KOH and filter papers. Equilibration was in the dark for ten minutes.

Results

Cyanide experiments. The results of three sets of experiments were clear-cut in the sense that, even at low concentrations, cyanide inhibited the respiration of fertilized eggs. Data from one of these are plotted in Figure 1. The lines labelled

Table I

The effect of cyanide, added 20 to 25 minutes after fertilization, on the percentage development of eggs of Urechis caupo, examined at 3 hours. The sea water contained 0.01 M glycyl glycine, pH 8.0, T° C. 20

Conc. HCN	Uncleaved	2-cell	4-cell	8-cell	16–32 cell	Unfertilized
10^{-4} M	99 } polar bodies					1
$5 \cdot 10^{-5}$ M	99 } polar bodies					1
10^{-5} M	49	30	20			1
$5 \cdot 10^{-6}$ M	2	1	4	46	46	1
0	3				96	1

O,KOH and O,$Ca(OH)_2$ were controls to compare the CO_2-absorptive powers of 10% KOH and $Ca(OH)_2$ in the center-wells of the manometer flasks. As this and other tests showed, the $Ca(OH)_2$ proved as effective as the KOH in absorbing CO_2 under the conditions of these experiments.

Table I shows the effects of the different concentrations of cyanide on the development of the eggs when examined at the end of the experiment.

Carbon monoxide experiments. The results of an experiment in which just-fertilized eggs were subjected to 95% CO/O_2 and 95% N_2/O_2 are shown in Figure 2, in which periods of illumination and darkness are indicated by black and white blocks along the time axis. If the rate of O_2-consumption [3] in the curve labelled CO/O_2 is examined by itself, it is clear that it rises upon illumination and falls in darkness in the manner considered characteristic of cytochrome-catalyzed respiration. When, however, comparison is made between the curve labelled CO/O_2 and the control labelled N_2/O_2, it is equally clear that, in the light, CO also stimulates the gas-uptake of these eggs. Illumination had no inhibitory effect on the O_2-uptake of eggs in equilibrium with air.

Table II shows the effect of CO in this experiment on egg development. The

[3] The use of the terms O_2-consumption, O_2-uptake, and respiration in the description and discussion of the CO-experiments is subject to the qualification that there is the possibility (see Discussion) that some of the gas consumed might be CO.

inhibition is not so marked as in the cyanide experiments, but it might, of course, be more dramatic if higher CO tensions were used.

The results of six sets of experiments with 95% CO/O_2 and 95% N_2/O_2 are presented in Table III. The last two columns of the table give a measure of the effect of CO on the respiratory rate, in the dark and in the light, based on lateral

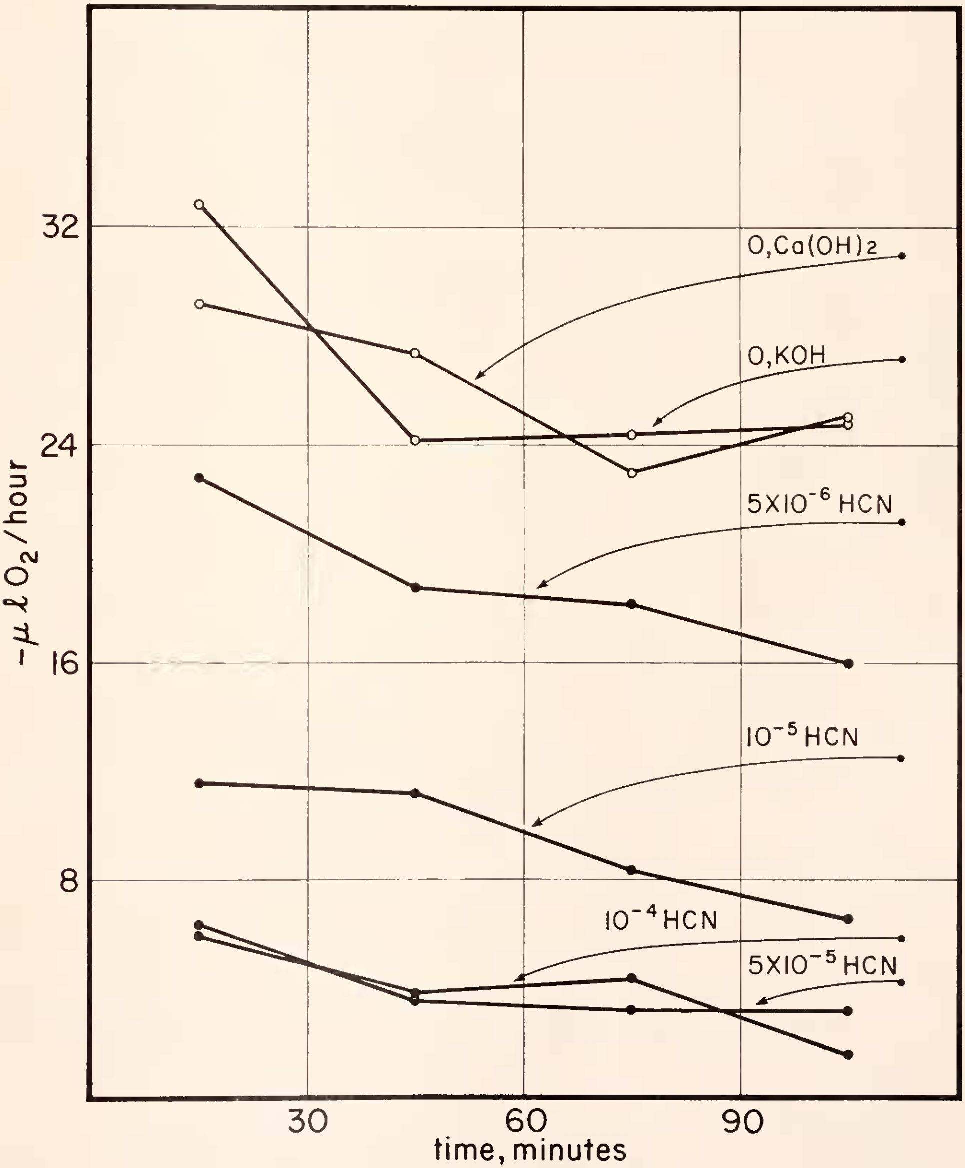

FIGURE 1. The respiration of eggs of *Urechis caupo* in the presence of HCN. For further details see text.

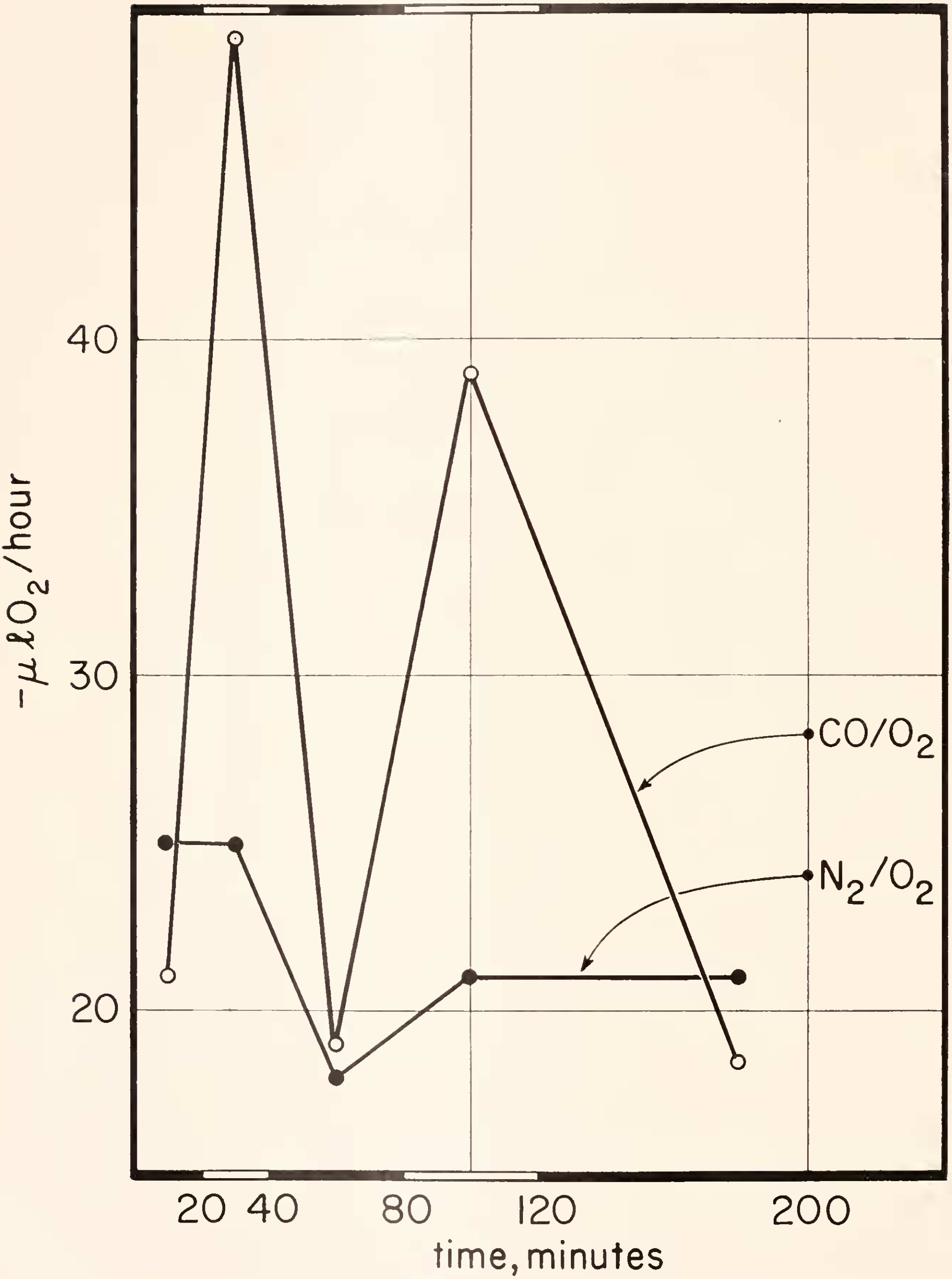

FIGURE 2. The oxygen uptake of eggs of *Urechis caupo* in the presence of 95% CO in O_2 and of 95% N_2 in O_2. The black and white blocks along the time axis correspond to periods of darkness and illumination. For further details see text.

TABLE II

The effect of 95% CO in O_2 and 95% N_2 in O_2 on the percentage development of eggs of Urechis caupo, exposed at ¼ hour and examined at 5 hours after fertilization. The sea water contained 0.01 M glycyl glycine, pH 8.0, T° C. 20

Gas	Uncleaved	64-cell	128-cell
Air	15	50	35
N_2	15	50	35
CO	15	85	

TABLE III

Effect of carbon monoxide on the respiration of eggs of Urechis caupo in the light and in the dark (All experiments started about 40 minutes after fertilization. Temp. 20° C.)

Experiment	Respiration period		Cu.mm. O_2 per hr. per 10^5 eggs		Ratios $\frac{\text{Resp. in 95\% CO–5\% } O_2}{\text{resp. in 95\% } N_2\text{–5\% } O_2}$	
			95% CO–5% O_2	95% N_2–5% O_2	Dark	Light
1	0′–15′	dark	7.6	9.7	0.78	
	15′–30′	light	14.6	9.7		1.59
	30′–45′	light	13.5	8.2		1.65
	45′–60′	dark	5.9	7.7	0.77	
	60′–75′	dark	4.9	6.7	0.73	
2	0′–20′	light	15.7	9.8		1.60
	20′–60′	dark	7.1	9.1	0.78	
	60′–100′	light	19.5	9.3		1.88
	100′–140′	dark	7.8	8.8	0.89	
	140′–160′	light	15.7	9.8		1.60
3	0′ 21′	dark	7.5, 7.6	9.4, 8.7	0.83	
	20′–40′	light	17.1, 18.3	8.0, 10.1		1.95
	40′–80′	dark	6.8, 6.8	7.2, 6.1	1.02	
	80′–120′	light	13.5, 15.1	6.9, 8.4		1.87
	120′–140′	dark	6.9, 6.6	8.0, 7.5	0.87	
4	0′–30′	dark	8.7	11.8, 10.8	0.77	
	30′–60′	dark	9.6	11.1, 14.7	0.75	
	60′–90′	light	18.4	8.2, 9.3		2.10
	90′–120′	light	17.6	8.8, 8.5		2.02
	120′–240′	light	15.2	8.9, 8.9		1.71
	240′–270′	light	14.4	8.9, 8.5		1.66
	270′–300′	light	15.2	10.4, 10.0		1.49
5	0′–90′	light	15.8	6.4		2.47
	90′–150′	light	13.0	5.7		2.28
	150′–180′	light	14.1	5.7		2.47
	180′–240′	light	13.8	6.5		2.12
6	0′–60′	light	13.5	9.4		1.44
	60′–210′	light	15.4	10.9		1.41
	210′–240′	light	13.5	7.4		1.82
	240′–270′	light	11.8	5.7		2.07
	270′–300′	light	15.2	9.9		1.53

comparisons (*i.e.*, of different vessels run in parallel with aliquots of the same egg-suspension). In the dark the respiratory rate in 95% CO/O_2 is consistently lower than in 95% N_2/O_2. Rigid statistical treatment would be complicated because of the differences in times of readings, magnitude of respiration, etc., in the different experiments. However, a simple averaging of the percentage decrease (with double and quadruple weights for experiments 4 and 3, respectively) gives a 15 per cent inhibition of respiratory rate in 95% CO/O_2 in the dark.

Similarly calculated, there is in these experiments, in the light, an 85 per cent average increase in respiratory rate of the eggs in 95% CO/O_2 over that of the parallel controls in 95% N_2/O_2. The figures in Table III also show, for individual manometer vessels, the great effect of alternate light and dark periods on the respiration of the eggs in 95% CO/O_2 and the lack of significant effect of light and dark periods on the respiration of the eggs in 95% N_2/O_2.

Spectroscopic examination of eggs. We have examined the unfertilized eggs of *Urechis* with a narrow-dispersion hand spectroscope (Keilin, 1925) at the temperature of liquid nitrogen, the eggs being suspended in 50% glycerol (*v/v*) with sodium dithionite added (Keilin and Hartree, 1939, 1949, 1955). A double absorption band at 551 mμ, which is in the region of the α-band of cytochrome *c*, could be clearly seen. A further, faint, absorption band at 580–590 mμ (cytochrome *a*) was also seen. The presence of these absorption bands was confirmed by Professor D. Keilin and Dr. R. Hill.

Reduced cytochrome *c* was rapidly oxidized by egg brei in phosphate buffer. A peculiar phenomenon was observed during examination of the oxidation of cytochrome *c* by egg brei. When the oxidized cytochrome *c* and egg brei was kept in comparative darkness and then illuminated through the microscope sub-stage condenser (which automatically occurs during spectroscopic examination), the absorption bands of reduced cytochrome *c* gradually reappeared. This also was confirmed by Professor D. Keilin and Dr. R. Hill. The most probable interpretation is that in the presence of light, some reducing substance is produced by the eggs, causing the reduction of cytochrome *c*. This phenomenon may have some connection with the inhibitory action of light on the respiration of sea urchin eggs (Rothschild, 1949), though, as mentioned above, we have not observed any comparable light-inhibition of respiration in *Urechis* eggs. Certain dyes are affected by light in ways which would be consistent with the observed reduction of cytochrome *c* in light, which raises the possibility that urechrome may be concerned in the phenomenon. For example, Equ. (3) in Clare's article in Hollaender's *Radiation Biology*, Vol. III (1956)

$$DH_2 + O_2 \rightarrow H_2O_2 + D,$$

if written in the form

$$DH_2 + 2\ \text{cyt.c}^{3+} \rightarrow 2\ \text{cyt.c}^{2+} + D + 2H^+$$

is suggestive in this connection.

Discussion

In the introduction to this paper, reference was made to Horowitz's (1940a) view that urechrome and not cytochrome catalyzed the respiration of *Urechis* eggs;

this opinion was based on the facts that urechrome is reversibly autoxidizable and that no absorption bands of cytochrome were observed. We have now shown that the absorption bands of cytochrome are present in these eggs and that an egg brei can oxidize reduced cytochrome *c*. Moreover, the inhibition studies with cyanide and carbon monoxide support the view that the respiration of these eggs is cytochrome-catalyzed. Just where urechrome fits into the picture is, at present, uncertain. The effects of CO and cyanide on this pigment have not, as yet, been studied.

The stimulating effect of carbon monoxide on respiration has been noted in many experiments with eggs and other tissues. The following citations from the literature on this subject will serve to illustrate the widespread occurrence of the phenomenon.

Runnström (1930) found that the respiration of unfertilized eggs of *Paracentrotus* and *Arbacia* was either not inhibited or somewhat higher in carbon monoxide-oxygen mixtures than in air, while that of the fertilized eggs was greatly inhibited. Presumably, although not explicitly stated, these experiments were run in the dark.

Lindahl (1939) obtained a 44% stimulation of the respiration of unfertilized eggs of *Paracentrotus* by 75% CO/O_2 in the dark, and this increased (to ca. 100%) upon illumination. With decrease in oxygen tension to 5% (+ 15% N_2 and 75% CO) the stimulation decreased. For freshly fertilized eggs in the dark he obtained a slight stimulation in 75% CO/O_2 and a marked inhibition in 95% CO/O_2. In the light the fertilized eggs showed marked stimulation by 75% CO/O_2 and this effect decreased as the O_2 concentration was dropped to 5% at constant CO.

Rothschild (1949) measured the respiration of unfertilized eggs of *Psammechinus miliaris* in various $CO–O_2$ mixtures. In 14 comparisons of the effect of 95% CO/O_2 with 95% N_2/O_2 in the dark there was no difference in two, an 11% decrease in three and a 14% increase in nine. Twenty-four comparisons of the effect of 95% CO/O_2 in dark with that in light showed a 44% increase in the light. At the same time he found an inhibitory effect of light on the respiration of the unfertilized eggs in air. This averaged 38% in 44 experiments. With 80% CO/O_2 in the dark there was an average of 55% increase in respiration above that in 80% N_2/O_2, and no significant change upon illumination.

In the ascidian *Phallusia mammillata* Minganti (1957) found an increase in respiration of the unfertilized eggs in 95% CO/O_2 in the dark and a further increase in the light. The fertilized eggs showed a 14% to 20% decrease in the dark, which is about the same degree of inhibition as in the present experiments, and an increase (up to 40%) in the light.

Bodine and Boell (1934) obtained CO-stimulation of respiration of diapause embryos of the grasshopper *Melanoplus differentialis* and no significant effect of light. A similar stimulation by CO was found by Wolsky (1941) in a bivoltine race of the silkworm *Bombyx mori*, but not (Wolsky, 1938) in pupae of *Drosophila melanogaster*. Wolsky (1938) attributes this difference to the pupal stage being one of great activity as compared with diapause. Schneiderman and Williams (1954) found that the respiration of diapausing pupae of the *Cecropia* silkworm was but slightly affected by high concentrations of carbon monoxide; further experiments (Harvey and Williams, 1958) demonstrated that a cytochrome system functioned in this material, the resistance to CO being accounted for by cytochrome oxidase being present in great excess relative to cytochrome *c*.

In non-embryonic tissue the most extensively studied examples of CO-stimulation of respiration were those first reported by Fenn and Cobb (1932a, 1932b) in skeletal and heart muscle of frog and rat. This stimulation occurs in the dark or diffuse daylight and, as shown by Schmitt and Scott (1934), is increased by strong illumination. Fenn and Cobb (1932b) adduced evidence to show that the CO was oxidized to CO_2 and this has been further substantiated by Clark, Stannard and Fenn (1950) by the use of isotopically labelled CO. The latter investigators (1949) also reported such oxidation of CO by the intact animal (turtles and mice).

In plants Daly (1954) obtained increases of about 20% to 30%, in 95% to 97% CO, with leaf tissue of the wild plum, *Prunus americana,* in the dark. From the results of experiments with labelled CO he concluded that the increased gas-uptake by the tissue represents a real stimulation of respiration rather than oxidation of CO to CO_2. He also found a rather high R.Q. (up to 1.33) for the extra gas consumed and therefore suggested that aerobic glycolysis was increased by CO to a greater degree than O_2-uptake. He cited cases of such stimulation of aerobic glycolysis by CO which have been reported in spinach (Ducet and Rosenberg, 1952 [4]), carrot (Marsh and Goddard, 1939), and rat retina and mouse [4] chorion (Laser, 1937).

The above-mentioned investigations indicate that the stimulating action of CO on respiration is of wide incidence in cells and tissues of animals and plants. In some cases (skeletal and heart muscle of frog and rat) there is strong evidence that the extra gas-uptake is due to the oxidation of CO. In others (plum leaves) it appears to be due to the stimulation of endogenous respiration. In the case of the fertilized *Urechis* eggs, and the other cases that have been cited above, the mechanism of the stimulating action of CO is, as yet, unknown and would constitute an interesting area of further investigation. For the present purpose the demonstration of a light-sensitive action of CO on the gas-uptake of the *Urechis* eggs serves to support the other evidence presented that a cytochrome system is operative in this material.

One of us (R.) is indebted to the Biology Division, the California Institute of Technology, for their hospitality during the course of these experiments. We are indebted to Miss Mary Jones for technical assistance.

Summary

1. The respiration and normal development of fertilized eggs of *Urechis caupo* are inhibited by low concentrtaions of HCN, 5×10^{-6} *M*. Known concentrations of HCN were established within the manometer flasks by the use of $Ca(CN)_2$–$Ca(OH)_2$ mixtures in the center-wells, with and without the appropriate amounts of NaCN in the egg suspensions.

2. The respiration of fertilized eggs was photo-reversibly inhibited by 95% CO in O_2. The inhibition of development was not so marked at this tension as in the cyanide experiments.

3. CO markedly stimulated the respiration of the eggs in the light. The occurrence of a similar action in the dark is presumed to account for the moderate degree of depression of respiration by CO in the dark.

[4] Daly (1954) cited a 1951 paper instead of the 1952 paper listed here; also he referred to chicken chorion whereas Laser (1937) refers to mouse chorion.

4. Spectroscopic examination of the eggs at the temperature of liquid nitrogen revealed absorption bands at 551 mμ and 580–590 mμ. Absorption bands at these wave-lengths are associated with the presence of cytochromes *c* and *a*.

5. An egg brei rapidly oxidized reduced cytochrome *c*, but intense illumination of the system reversed the process.

6. It is concluded that the respiration of *Urechis* eggs is cytochrome-catalyzed.

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